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DEPARTMENT OF THE ARMY
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The physical basis of microscopic photometry

By R. Herrmann[1]:

[1] Paper read before the meeting "Biochemical Analysis 70", Munich, April 29, 1970.

Introduction: In the broadest sense what is meant by photometry is the measurement of radiation. We will limit ourselves at present to radiations in the visible and in the ultraviolet region of the spectrum. Even for this narrow aspect of the subject there are a large number of possibilities as to the manner in which chemical information on the substance is arrived at from the change in incident light due to reciprocal interaction with the substance to be examined. It should be recalled that there are absorption methods, fluorimetric methods, interferometric methods, etc. All these procedures, with a few limitations, can be applied to microscopically small objects. In such cases, for the study of such small objects, a microscope will also be used. This is the sense in which we speak of microscopic photometry. Because of the large number of possibilities we will limit ourselves to processes relating to absorption on the microscopic scale in our discussion of fundamentals.

The advantage of microscopic photometry is that it is possible to arrive at concrete information regarding the chemical composition of individual drops, individual cells, individual organelles in cells, etc., while the usual chemical procedures can only provide mean values of combined masses of many different particles, each with a different previous history, different age, different shape, etc.

The first to give a strong impetus to the special methods of microphotometric absorption was T. Casperson, 1936 (1, 2).

Physical foundations. For the mathematical description of the usual absorption process the so-called Bouguer-Lambert-Beer law is used:

$$E_\lambda = \varepsilon_\lambda . c . d,$$

with $E = \log \frac{I_o}{I}$.

In this E_λ means the extinction measured, ε_λ a material constant

dependent upon the wave length, c the concentration sought, and d the layer thickness of the cuvette (3), I_o the intensity of the incident radiation, I the radiation weakened as a result of absorption.

I should like to stress emphatically that this law is not a generally applicable law of nature; it only applies within certain limits, notably for the most part only at low concentrations and a medium kept constant (temperature, pH, etc.).

Fig. 1 gives an idea of the principle of the process of absorption. In order to detect absorption in such small volumes it is necessary for the measuring pencil of rays to go through a microscope. The optical separation of the beam actually used or of the measuring surface generally only takes place in the actual picture plane of the microscope objective. The frequently used beam diameters or measuring surfaces ΔF, related to the preparation, are at $0.5\mu m^2$. The absorption processes on the micro scale can in principle be described by the same mathematical formulae as in the macro range, that is to say by the Lambert-Beer law. However, on the micro scale there are a few additional features. The most important of these will be described below.

Aside from these quantitative relationships one can naturally use the wave length-dependent absorptions, considered only qualitatively, for the identification of substances. This applies both to the macro and the micro range.

Unknown layer thickness. Since in the case of cuvettes the layer thickness "d" is known, with the Lambert-Beer law it is possible to determine the concentration "c" sought in the macro range by measurement of the extinction E. Transferring this idea to the micro range, however, the basic difficulty arises that the layer thickness "d" is usually not known. It could only be determined with additional losses and additional errors. There is the added fact that the layer thickness of cells - and incidentally also of drops - differs locally over the entire object, quite in contrast to cuvettes. Basically speaking, upon transferring the macro process to the micro range one only has one measuring value, extinction E, and two unknown ones, c and d, in other words one unknown more than before. Furthermore d is not even properly definable. This difficulty can be overcome by changing the Lambert-Beer law by appropriate mathematical transformation into a form in which it is no longer the concentration, that is the mass per volume unit, which appears, but rather the so-called surface covering. All masses are

to some extent projected onto the base, for instance onto the surface of the slide. Now the projected masses are determined per surface element, or this is equally integrated over the entire projected cell surface or the entire projected drop. This summation or integration of all mass elements in the final analysis leads to a total mass M of the cell or drop absorbing at the appropriate point of the spectrum. In microscopic photometry, therefore, one must not ask for concentration c, but one must determine M masses. The result of this transformation (4, 5) is:

$$\Sigma E_\lambda \cdot \Delta f = \epsilon_\lambda \cdot M,$$

with ΔF = surface element.

Selection of the measuring surface or diameter of beam.

From the transformed equation one can read off a measuring procedure directly, that is here in the micro range, with a variable layer thickness and inhomogeneous distribution of material, one can not simply make an integrating absorption measurement over a larger volume, for instance an irradiated cell or a drop and get a mean extinction from it - which is entirely permissible in the case of cuvettes with a definite layer thickness and homogeneous content - but must measure surface element after surface element successively and add together or integrate the extinctions obtained or the masses belonging to them. The need for this different procedure is due to the fact that summation or the calculation of a mean on the one hand and using logarithm to get extinctions on the other are not interchangeable operations.

The size of the measuring surface or of the light beam used, ΔF, is laid down in the case of such measurements by the following requirements: the diameter of the measuring beam on the one hand must be so small that no appreciable differences in thickness should occur within the surface element belonging to it or the volume element of the cell or drop envisaged above it (negligible distributional error), but on the other hand the beam must be selected large enough to remain above the limits of the power of resolution of the microscope and one should receive sufficiently high, still just accurately measurable intensities (accurate radiation capacities) through this beam. It should be noted that with too low a radiation capacity the amount of electronic noise in the signal becomes too large, that is the needles on the measuring instruments are agitated and thus become inaccurate (6,7).

Scanning the object. From the transformed Lambert-Beer law one obtains, for example, the procedure for measuring the microscopic object - for instance a cell or a drop - with a monochromatic beam of rays having a small diameter of the beam with a surface of, e.g., $0.5\mu m^2$ ($\Delta f = 0.5\ \mu^2$), surface element by surface element, individually and successively with regard to the partial extinction achieved, and for adding together the partial elements, multiplying them with the scanned surface and next dividing by ε_λ, the material-specific extinction coefficient. The result of this measuring and calculating operation, in accordance with the transformed Lambert-Beer law, then gives the mass of absorbing material which is being sought in the microscopic object, for instance the nucleic acid content in g in human sperm. There are apparatuses that can deal with the measuring operation referred to and the subsequent calculations completely automatically. Such quantities of nucleic acid in the example cited are often the size[2] of a few pg (8, 9, 10), while the limit of detection of the method in good apparatuses is 10-100 fg (9).

[2] 1 pg (picogram = 10^{-12}g; 1 fg (fentogram) = 10^{-15}g.

The scanning mentioned above, as has already been stated, is necessary because the microscopic object does not have a layer thickness that remains the same, as for instance the solution in a cuvette when using the macro method.

The scanning of the object can take place in different ways: Fig. 2 shows a few examples. For literature see l.c. (11). I should like to point out that in the measuring procedure with rows, measuring surfaces that are far outside the microscopic object do not cause any errors in measurement provided the area around the object is free, that is to say shows an extinction of 0. Such measuring procedures are thus used predominantly in the case of isolated cells or free-hanging drops in microanalysis etc. When measuring cells in groups, as for instance in a histological section, additional difficulties arise due to the area surrounding the cell in question. This can to some extent be overcome by preprogramming the area of the whole preparation which is to be scanned - for instance the cell about to be measured - into the apparatus with a view to its boundaries and to some degree give the apparatus the task of only scanning this area completely automatically, but not the surroundings. In the case of simpler devices this can be done manually. It should be mentioned in this connection that even in the case of microscopic objects that lie free, errors may arise due to the fact that the parts at the edge of the cell, according

to the scanning and evaluating procedure used, are measured at the same time to a varying degree. Details of this may be found in the literature (12).

In this connection we should also like to mention that aside from possibilities of variation in the scanning there are three basically different procedures for accomplishing this successive measurement of the individual surface elements of a cell or a microscopic object:

1. The measuring beam of rays is kept still and the microscopic object is moved intermittently or continuously row by row according to one of the scanning methods indicated above (fig. 3, a-c).

2. The object is kept still and the pencil of rays is moved accordingly on the picture side, for instance with the aid of a movable deflecting mirror.

3. Again, the object is kept still but the scanning pencil of rays is moved on the illuminating side, for instance with the aid of a flying spot.

The procedure named under 1 represents considerable expenditure from the standpoint of the construction of the apparatus. However, it has the advantage that the measuring accuracies that can be achieved with it are generally better than with the second and third procedures. For one cannot simply postulate that the visual field of a microscope will be sufficiently evenly illuminated to guarantee a constant 100% permeability value or a constant initial extinction value ($E = 0$) over the entire surface. Errors that may arise due to these inevitable inadequacies in all optics can be disregarded in case 1, because measurement always takes place at the same point of the measuring field.

The plug measuring procedure. With the relatively exact scanning processes just described, it was stipulated, owing to the varying thickness of the microscopic object, that the object be divided up into individual small volumes or projected measuring surfaces ΔF belonging to them, to keep undesirable effects of these differences in thickness and material distribution on the results of measurements as small as possible. There is, however, a so-called single plug measuring process (not to be confused with the above-mentioned multiple plug measuring process), which to some extent overcomes these obstacles. The nucleus or the microscopic object are measured in one operation and the mean extinction is

(almost inadmissibly) determined and with it the total mass of the absorbing substance belonging to it (fig. 3). Various authors have had some controversy regarding the errors that can arise as a result of such simplified procedures (8). It has been found in these studies that in the case of similar drops, that is to say of about the same size and shape, or in the case of not too inhomogeneous cells, very exact results can be obtained in the middle position of the cell, if one takes care not to let the border portions of the cell, with greater differences in thickness and material distribution, be included in the measurement (limiting the plug). This is possible by appropriate limitation of the measuring beam of rays to the middle portion (fig. 3).

Procedures of this kind have the great advantage that expenditures for apparatuses are considerably smaller than with the scanning method. The plug methods, however, require greater critical faculties on the part of the investigator in the selection of the diameter of the measuring beam in relation to the size of the cell. Furthermore it is difficult to extrapolate from the measured partial volume to the whole volume. For this reason the two-wave length measuring process is used for preference to eliminate inhomogeneity of the object (see p. 326).

Small layer thickness, large extinction coefficient. If we bear in mind the relations of the Lambert-Beer law described at the beginning, or the law transformed for microscopic photometric investigations, the following must be taken into account: On the micro scale, when making measurements on human cells, one works with layer thicknesses that will be around 5-10μm. On the macro scale one works mostly with a 10 mm layer thickness, that is the layer thickness is smaller in the micro method by a factor of about 1000-2000 than in the macro range. If we postulate the same substances and same substance concentrations and same extinction coefficients in the macro and micro range, it follows that on the micro scale the extinctions measured will be smaller than the normal by at least a factor of 1000. This, however, also means that most extinctions on the micro scale are no longer measurable or only with moderate accuracy. Thus one can only measure substances which have an extremely high extinction coefficient with adequate accuracy, without additional staining operations, directly in the biological cell or in the micro object. Few substances fulfill this condition in the case of cells. Among them are primarily the nucleic acids, and also a few protein fractions. Mention may also be made of the derivatives of hemoglobin, some pigments and

respiratory enzymes. Fig. 4 shows the course of the extinction coefficient for some of these substances. Had the nucleic acids, which have been the focus of interest in recent years, not been included, microscopic photometry of unstained preparations would scarcely have attained its present importance.

If we wish to measure, in addition to substances endowed by nature with high extinction coefficients, other substances in the cell or in the microscopic object, special chemical preparative reactions must take place, which bring out a new substance with a sufficiently high extinction coefficient from the biochemical compound with an inadequate natural extinction, for instance by histochemical staining (incorporation of chromophoric groups into weakly absorbing substances). Table 1 shows the color reactions frequently used today in microscopic photometry.

A few other possibilities exist. Unfortunately it is difficult to regulate these chemical or enzymatic reactions in such a way as to give the same color or extinction for the same concentration or amount of substance sought in all cases, that is to say with a different biochemical milieu, different pH, different fixation, different preparation with different surface structures, etc. These difficulties will only be referred to briefly, without entering into them in detail. In this connection reference is made to the literature (13). It should also be mentioned that by a resourceful combination of the chemical and enzymatic reactions one can arrive at more specific data with the absorption processes mentioned above. There are also so-called double staining methods which do not disturb each other's dye release when using the appropriate technique and allow detection of two different chemical substances side by side, for instance DNA and RNA (14). Thus for instance the nuclear substances, which essentially consist of desoxyribonucleic acid and ribonucleic acid, can be treated with ribonuclease between the first and second measuring operations, and therewith, by making use of the differences, one arrives at more specific data. My basic reservations when using microchemical reactions on cells also apply to such elutions. Reference is made to chemical extractions with acids or blocking reactions for the (partial) prevention of some reactions.

Validity of the Lambert-Beer law. As was mentioned in the introduction, the Lambert-Beer law normally only applies to small concentrations roughly below 100 nM. In biological tissue, however, there are often very high concentrations, for instance of nucleic acid, in the cell nucleus. If the concentrations were not so high, measurements could scarcely be made. The nuclear substance

consists almost exclusively of nucleic acid and nucleoproteins. One must therefore raise the question whether the Lambert-Beer law still applies under these extreme conditions. In the case of nucleic acid this has been proved with the aid of purest synthetic preparations in Svensson's model experiment (15).

The question still remains, however, whether this model experiment can unconditionally be transferred to every type of nucleic acid with possibly different biological admixtures. The difference in the preparation of samples, for instance the fixation processes, should also be included in these considerations. It should also be stated that corresponding studies for the dyes shown in table 1 have not so far been made on a systematic basis. It may be assumed that divergences exist from the Lambert-Beer law at the high concentrations and extreme milieu in question. A number of systematic comparative experiments have been made on the nucleic acid concentration measured without UV coloring on the nucleic acid absorption band at 260 nm on the one hand, and the nucleic acid content determined with Feulgen staining and measured in the visible region on the other (10, 16). As a rule in such comparative experiments only moderate correlations were found between the results obtained by the two methods. The divergences or moderate correlations have so far been attributed to the different dye-binding capacity of the various biological materials (13). We are of the opinion that such causes of error should definitely not be rejected out of hand and require further systematic study. On the other hand there also exists the possibility that these divergences can be partly or completely attributed to divergences from the Lambert-Beer law on both sides of such comparative experiments. The "proof" so far produced, generally biologically, for the satisfactory working of one or the other method cannot be accepted as strict scientific proof.

Two-wave length process for the determination of two components. For the sake of simplicity the absorption methods were represented here as though only one absorbing substance took part in the formation of the measuring value. In practice one will generally be dealing with two or more absorbing substances simultaneously and the absorption of these different substances will be superimposed upon one another. Fig. 5 shows an example of a two-substance mixture A and B.

For the next step, for the sake of simplicity we will postulate that only two absorbing substances A and B take part simultaneously,

and that these have a mass of M_A and M_B in the drop or cell studied. Inhomogeneity - in contrast to the next section - will not have a disturbing effect. It will further be postulated that with the given apparatus it is possible to measure at two suitably selected points (see fig. 5) of the spectrum λ_1 and λ_2, and also that the twice two extinction coefficients ε_{A1}, ε_{B1}, ε_{A2}, ε_{B2} are also known (index A and B relates to the substance, index 1 and 2 relates to the wave length). With these postulates one can set up a system of equations with the two unknowns M_A and M_B, as follow

$$\sum E_1 \Delta f = \varepsilon_{A_1} \cdot M_A + \varepsilon_{B_1} \cdot M_B$$
$$\sum E_2 \Delta f = \varepsilon_{A_2} \cdot M_A + \varepsilon_{B_2} \cdot M_B$$

The solution of this system of equations expressed as determinants gives:

$$M_A = \frac{\begin{vmatrix} \sum E_1 \Delta f & \varepsilon_{B_1} \\ \sum E_2 \Delta f & \varepsilon_{B_2} \end{vmatrix}}{\begin{vmatrix} \varepsilon_{A_1} & \varepsilon_{B_1} \\ \varepsilon_{A_2} & \varepsilon_{B_2} \end{vmatrix}}$$

bzw.: or:

$$M_B = \frac{\begin{vmatrix} \sum E_1 \Delta f & \varepsilon_{A_1} \\ \sum E_2 \Delta f & \varepsilon_{A_2} \end{vmatrix}}{\begin{vmatrix} \varepsilon_{A_1} & \varepsilon_{B_1} \\ \varepsilon_{A_2} & \varepsilon_{B_2} \end{vmatrix}}$$

Corresponding systems of equations can also be set up for 3 and more absorbing substances, provided one can measure the various absorptions on 3 or more different wave lengths and knows the corresponding extinction coefficients. The record of absorption bands superimposed upon one another determined in this manner, so far as we know, is 15 substances in the serum, but obtained in the macro region (17). For microscopic photometry these processes have the following significance:

a) Mutually superimposed absorptions, for instance of nucleic acid and protein, can be separated and recalculated for the pure nucleic acid content or protein content. The same applies to superimposed absorptions after application of double staining methods (see below).

b) With this multi-wave length method under certain conditions one can eliminate unspecific but nevertheless wave length-dependent influences, for instance the effects of stray light, mathematically since these behave like a pseudo-absorption (see below).

Possibilities for errors due to scattering, bending, refraction and reflection.

We have already referred to certain difficulties in measuring in the micro range and of possibilities for their elimination. Within the framework of this general report it is difficult to give an overall account of all possible disturbances and possibilities for their avoidance. However, we would like to refer to two other sources of error that seem to us important and how to avoid them: aside from the different layer thickness of the cell and apart from the inhomogeneous distribution of substance within the cell and aside from errors in focusing, one also has to bear in mind that scattered particles (by scattered we also mean the deflection of rays by bending and reflection in the following) in the volume to be measured or particles that happen to be above or below and even outside the volume irradiated by the measuring beam of rays can deflect the radiation more or less laterally. This can give the impression of an additional pseudo-absorption, because radiation is lost in the direction of measurement. These pseudo-absorptions have nothing to do with the absorption to be measured (fig. 6).

On the other hand scattered radiations can fall in from the border portions which apparently reduces the absorption in the measuring beam of rays (reduction of contrast). With the above-mentioned multi-wave length method it is necessary to some extent to determine the pseudo-extinction coefficients of these pseudo-absorptions, due to these effects. This involves a basic difficulty, because it is not possible to build up the biological preparation with its complicated composition from its individual parts while under observation. It is known that this scattered radiation must increase with the shortening of the wave length. For certain mathematically defined particle sizes and shapes and differences in refraction indices the ratio of scattering can be calculated theoretically or extrapolated from absorption-free regions of the wave length (21, 22). This can in fact be utilized to deduce information as to form and size of the particles from the ratio of scattering (23). In the case of the biological object with its complicated composition, however, there is the basic difficulty that little can be done with these mathematical models, because in practice one seldom has to do with homogeneous particle sizes and particles shapes, or indeed usually with homogeneous differences in refraction indices, and furthermore these parameters even differ from one preparation to the other. Undesirable effects of this kind on the results of measurement can, as a rule, be kept small, however, by suitable preparative techniques on the one hand and by a suitable microscopic illuminating technique

on the other. Freeze-drying and the placing of the material in an UV-permeable material with a relatively high refraction index adjusted to the tissue substance, for instance glycerin, as recommended by Caspersson (2, 24) is mentioned as one of the techniques for reducing scattered light. For a suitable illuminating and observation technique again reference is made to Caspersson's recommendation (1, 25) to work with a small condenser aperture of about 0.2-0.3 with a small light field diaphragm, on the other side of which the observation aperture should, on the other hand, be chosen as large as possible, for instance 1.0 (fig. 7). When measuring (not when adjusting the preparation), the light field diaphragm should be narrowed to the size of the measuring beam of light actually being used.

Calibration process. In principle if we know the extinction coefficient one can conclude the mass of the absorbing substance in the cell, the drop, etc., directly from the measured extinctions, using the equations given above. One can seldom rely upon absolute procedures of this kind; because the possibilities of error referred to as well as others can give rise to absolue errors that are difficult to ascertain. For this reason relative procedures are generally preferred, that is to say procedures in which the measuring value is obtained not absolutely, but relatively in relation to a comparative preparation studied under the same conditions. The process with working units as relative measuring value has become widely accepted (13, 26, 27). For instance in the study of human sperm, bull sperm is often used, in some cases added to the human sperm in the preparation (28). This is suitable for "calibrating standards" because the biological variation of its nucleic acid content etc. is very small, in other words almost constant. The draw back of some calibrating instruments is only that with the comparative measurements on a single cell one does not have sufficient information in such an instrument, but is forced to determine a statistical mean from a larger number of cells.

Sernetz and Thaer have presented an interesting and very accurate calibrating process developed by them for fluorescence analysis (29). We are convinced that this elegant calibrating method can also be used for the absorption analyses discussed here if one proceeds in the appropriate manner.

Instrumentation. In the early days of microscopic photometry one was forced to work with microscopic photometers of one's own devising. We ourselves have been active in this area for some time (30). Meanwhile the optical industry has taken up this special problem of instrumentation and there are now a number of commercial devices available for almost every application. As to the selection

of such instruments I should like to say the following: The more convenience one requires when carrying out the measurements, the more expensive the the supplies will generally be. This increase in costs caused by automation and computer technology is naturally only worthwhile if one subsequently also has to study corresponding quantities. I personally, therefore, am of the opinion that a resourceful and critical investigator can arriave at absolutely acceptable results with relatively simple means within a reasonable time, provided that he is not overwhelmed with large series of measurements.

Outlook. Microscopic absorption methods in the visible and ultra violet region of the spectrum have acquired serious competition from fluorescence analyses of microscopic preparations. I scarcely believe, however, that the fluorescence method will be able to displace the absorption methods completely. It is merely a matter of recognizing the advantages and disadvantages of the two methods and to select the best method for a certain purpose. So far as the absorption methods alone are concerned, we believe that in the course of time further possibilities for their application will be opened up by the disclosure of more (native) absorptions in short wave UV and by new methods of staining; also that the results, so far not always satisfactory, following such staining will be improved in the near future when a better understanding is gained of the irregularities arising in the staining methods, thus making better reproducibility possible. Physical and technological progress in the development of simpler and more efficient, mostly automatic, devices will also bring further support for these absorptions methods.

Legends.

1. Measuring device for microscopic (spectral) photometry. The diameter of the masuring beam of rays effective for measurements, with surface Δf in the preparation, appears enlarged in the picture plane of the microscope, generally represented by a diaphragm. I_o appears behind the diaphragm when the microscope is focused onto an empty space of the preparation. Correspondingly I appears after focusing on something like an absorbing cell.1. Picture plane. 2. Diaphragm. 3. Source of radiation.

2. Possibilities for scanning of a microscopic object: a) Multiple plug measuring procedure, that is break up of the beam of light or measuring dot. b) Systematic surface to surface procedure (time succession of the measuring beam of rays. c) Meandering shaped continuous measurement (row-by-row scanning of the preparation). d) Measurement with the aid of a Nipkow disk (similar to c, but not with straight rows, all rows penetrated in one direction.

GRAPHS

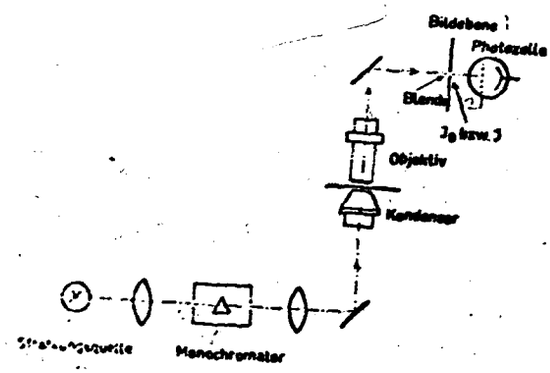
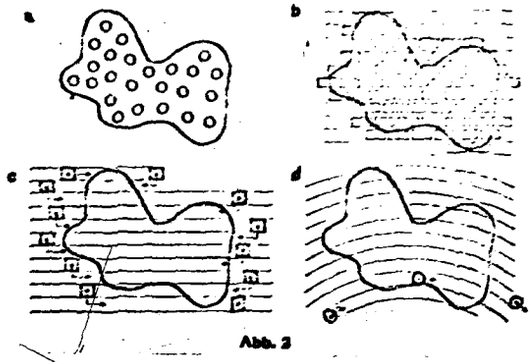


Abb. 1



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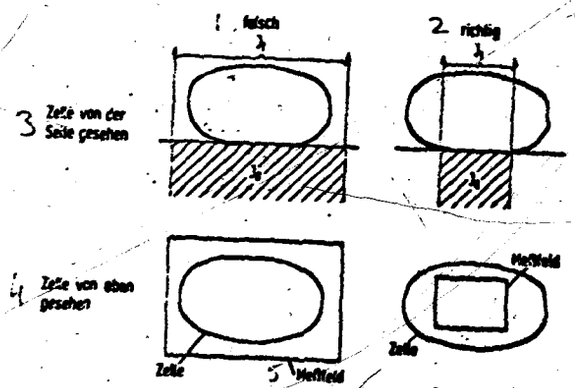

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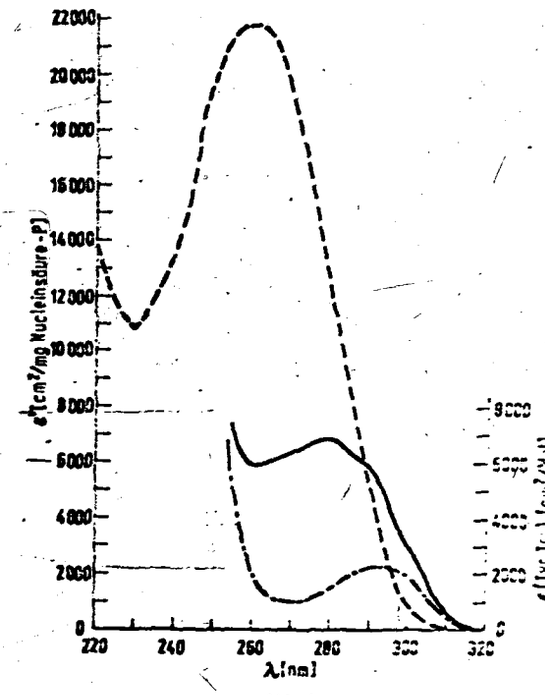

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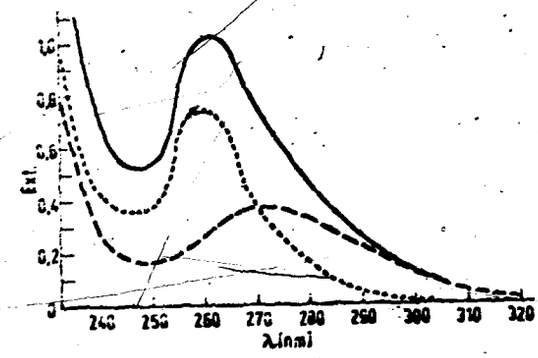

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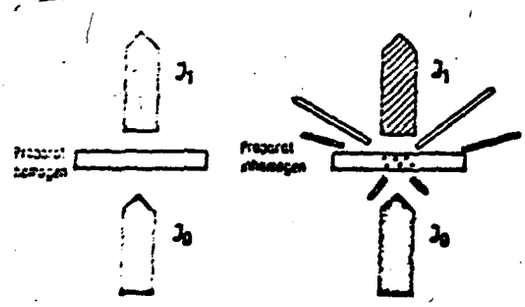

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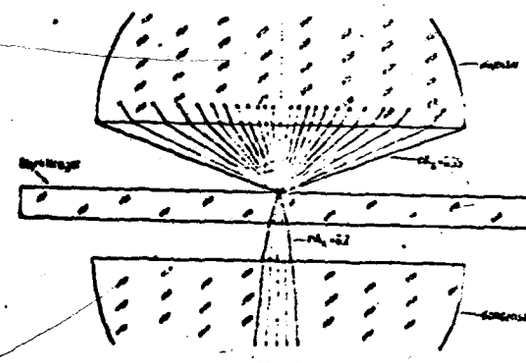

Abb. 7

3. Plug measuring process (schematic), Left 2 diagrams, measuring beams of rays too large in comparison to the micro object. Right 2 diagrams measuring beams considerably smaller than the object (correct) 1. Wrong. 2. Right. 3. Cell seen laterally. 4. Cell seen from above. 5. Measuring field.

4. Spectral course of the extinction coefficients of a few substances with sfficiently strong absorption. Ordinate on the right applies to tyrosine (-.-.-) and tryptophane (———)/ Left ordinate applies to nucleic acids (----), for which because of the difference in molecular weights one generally only gives relative values (Acc. to Sandritter, 5). 1. Nucleic acid.

5. Superimposition of two absorptions (schematic). The .... curve represents a nucleic acid absorption, the ---- curve a protein absorption. Nucleic acid absorption (...) and protein absorption (----) add together to spectrum (———).

Table.

1. Histological staining methods used for microscopic photometry.

Color reaction	Color reaction suitable for	Maximum extinction nm
Feulgen reaction	DNA	570
Gallocyanine chrome alum	RNA + DNA	575
Millon reaction	Tyrosine	495
Millon reaction	Tyrosine and Tryptophane	365
Sakaguchi reaction	Arginine	510
Fast green staining	Histone protein	635'
Naphthol yellow staining	Total protein	435
DMAB-Nitrite reaction	Tryptophane	590

Extraction and blocking processes

Color reaction	extraction medium	suitable for
Fast green	HCl or H_2SO_4 or NaCl	Histone protein or acid-soluble proteins
UV measurement, dir.	Ribonuclease	RNA
Fast green	Blocking of the ϵ-amino-groups of lysine, then fixation again in formalin.	Lysine-portion of histones.

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